Detection of enteroaggregative *Escherichia coli* in faecal samples from patients in the community with diarrhoea

Claire Jenkins,¹ Mathias Tembo,^{2,3} Henrik Chart,¹ Tom Cheasty,¹ Geraldine A. Willshaw,¹ Alan D. Phillips,³ David Tompkins⁴ and Henry Smith¹

Correspondence

Claire Jenkins

claire.jenkins@royalfree.nhs.uk

¹Laboratory of Enteric Pathogens, Health Protection Agency, 61 Colindale Avenue, London NW9 5HT, UK

²Tropical Diseases Research Centre, Microbiology Unit, PO Box 71769, Ndola, Zambia

³University Department of Paediatric Gastroenterology, Royal Free Hospital, Pond Street, London NW3 QG, UK

⁴Yorkshire and Humber, Leeds Health Protection Agency Laboratory, Bridle Path, York Road, Leeds LS15 7TR, UK

The aim of this study was to assess the usefulness of a multiplex PCR assay targeting the *aat*, *aaiA* and *astA* genes for the detection of typical and atypical enteroaggregative *Escherichia coli* (EAEC) in bacterial cultures from faecal samples from patients with community-acquired diarrhoea. The isolates harbouring these genes were also tested using the HEp-2 cell-adhesion assay to clarify their EAEC status. *aat*, *aai* or *astA* was found in *E. coli* faecal isolates from 39 (7.8%) of 500 patients, and 20 of these strains adhered to HEp-2 cells in a pattern characteristic of EAEC. Eight isolates carrying the *aai* or *astA* gene but not the *aat* gene were shown to be HEp-2 cell test positive, although 12 strains with this genotype were HEp-2 cell test negative. Using the HEp-2 adhesion assay as the gold standard, the addition of primers detecting *aaiA* and *astA* to the *aat* PCR increased the number of EAEC isolates detected, but identified strains of *E. coli* that were not EAEC. The variety of genotypes exhibiting aggregative adherence highlights the problems associated with developing a molecular diagnostic test for EAEC. This PCR assay detects a variety of strains exhibiting characteristics of the EAEC group, making it a useful tool for identifying both typical and atypical EAEC.

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INTRODUCTION

Enteroaggregative *Escherichia coli* (EAEC) is the most commonly isolated pathogenic *E. coli* group from patients with diarrhoea in England (Tompkins *et al.*, 1999; Food Standards Agency, 2000; Wilson *et al.*, 2002), and has emerged as an important pathogen in travellers' diarrhoea (Adachi *et al.*, 2001; Wilson *et al.*, 2002), diarrhoea among children (Huang & Dupont, 2004) and in immunocompromised patients (Gassama-Sow *et al.*, 2004). EAEC are defined by their ability to adhere to HEp-2 cells in an aggregative adherence (AA) pattern, described as a 'stacked-brick' formation (Nataro *et al.*, 1987; Law & Chart, 1998). However, the HEp-2 cell adhesion assay is unsuitable as a diagnostic tool, as it is labour intensive

and requires experienced microbiologists to interpret the results (Nataro, 2005). A PCR method based on the presence of essential virulence factors would improve the diagnosis of EAEC disease.

Certain strains carry a high-molecular-weight plasmid (pAA) associated with AA (Vial *et al.*, 1988; Law & Chart, 1998), on which a number of virulence genes are located, including an antiaggregation protein transporter gene (*aat*; previously referred to as CVD432 or the AA probe) (Baudry *et al.*, 1990; Nishi *et al.*, 2003) and an enteroaggregative heat stable toxin (EAST) gene (*astA*) (Savarino *et al.*, 1991; Menard & Dubreuil, 2002). Located on the chromosome, on the pheU pathogenicity island, are the gene clusters SciI and SciII. SciII comprises a cluster of ORFs under the control of AggR, designated the AggR-activated island (*aai*). The contribution of the *aai* genes to EAEC pathogenesis is currently unknown, but these genes do not appear to play a role in adherence (Harrington *et al.*, 2006).

Abbreviations: AA, aggregative adherence; DA, diffuse adherence; EAEC, enteroaggregative *Escherichia coli*; EAST, enteroaggregative heat stable toxin; HPA, UK Health Protection Agency; LA, localized adherence; LEP, HPA Laboratory of Enteric Pathogens.

PCR assays that target genes located on the pAA, including *aat*, *aap* and *aggR*, have been described (Schmidt *et al.*, 1995; Cerna *et al.*, 2003). However, EAEC are a heterogeneous group, and not all strains that adhere to HEP-2 cells in a stacked-brick formation harbour the pAA plasmid (Cobeljic *et al.*, 1996; Itoh *et al.*, 1997; Dudley *et al.*, 2006). Alternative target genes that would identify both pAA-positive and -negative EAEC strains have been sought (Gioppo *et al.*, 2000; Elias *et al.*, 2002), but EAEC-specific genes characteristic of both groups have not been identified. Work carried out in this laboratory has shown that the *aaiA* and *astA* genes are potential targets for detecting both groups (Jenkins *et al.*, 2005, 2006).

In this study, a PCR assay targeting the *aat*, *aaiA* and *astA* genes was used to detect EAEC in faecal samples from patients with community-acquired diarrhoea. Strains harbouring one or more of these three genes were assessed for their ability to adhere to HEP-2 cells to confirm their EAEC status. The aim of the study was to assess the usefulness of the PCR for detecting typical and atypical EAEC.

METHODS

Faecal samples. Five hundred faecal samples, one per patient, were collected at the Leeds Health Protection Agency (HPA) Laboratory between May and August 2003. These samples were sent to the laboratory by general practitioners (GPs) in the Leeds area and were from patients with community-acquired diarrhoea who had visited their GPs. Of the 500 patients included in the study, 202 were male and 289 female. Information on the patients' gender was unavailable for nine of the samples. Four hundred and three samples were from adults (18 years old and over), 91 were from children (younger than 18 years old), and the patient's age was not recorded for six of the samples. Details of recent travel abroad (in the last 12 months) were noted for 57 patients, who had visited 33 different destinations. The countries visited most frequently included Spain (7), India (6) and Pakistan (5).

The samples were analysed at the Yorkshire and Humber Leeds HPA Laboratory for routine enteric pathogens. The pathogens detected included *Clostridium difficile* (6), *Campylobacter* species (12), *Salmonella* species (10), *Shigella sonnei* (2), *Shigella flexneri* (1), rotavirus (7), small round structured virus (2), *Entamoeba coli* (1) and *Entamoeba histolytica* (3). The appearance of each sample was noted and recorded as formed (58), semi-formed (332) or liquid (109). This information was not recorded for one of the samples.

These faecal samples were forwarded to the HPA Laboratory of Enteric Pathogens (LEP) on a daily basis, except on Fridays, to avoid the samples being in the post over the weekend. The samples were packed on ice until processed by LEP staff on the day of receipt.

Bacterial strains. The control strain used for the PCR reactions was an EAEC of serotype O44:H18 (042), described by Nataro *et al.* (1987). The following *E. coli* strains were used as control strains in the HEP-2 adherence assays: EAEC strain 042 (Nataro *et al.*, 1987) for the enteroaggregative phenotype, enteropathogenic *E. coli* strain E2348/69 (serotype O127:H2) (Scotland *et al.*, 1989) for the localized adherence (LA) phenotype, and the diffuse adherence (DA) control was a clinical isolate from a 13-month-old female admitted to hospital in the East End of London with acute diarrhoea, who had no other pathogens detected. *E. coli* DH5 α , a strain of K-12 that does not cause diarrhoea in humans and has no specific pattern in adherence assays, was used as a negative control.

Identification by PCR of bacterial isolates containing *aat*, *aaiA* or *astA* from faecal samples. The faecal cultures were plated onto MacConkey agar and incubated overnight at 37 °C. Nutrient broths were inoculated with a sweep of mixed colonies from the MacConkey plates, incubated at 37 °C for 2–4 h, and examined for the *aat*, *aaiA* and *astA* genes by multiplex PCR. Two microlitres of broth culture were added to the PCR mix, which consisted of 1 $\times$ PCR buffer, 200 μ M dNTPs, 1 μ M each of *aat*, *aaiA* and *astA* forward and reverse primers, and 0.75 U Hotstar *Taq* (Qiagen). PCR-grade water was added to make a final reaction volume of 25 μ l. The primers used in the PCR reactions have been described previously (Schmidt *et al.*, 1995; Elias *et al.*, 2002; Jenkins *et al.*, 2006), and are shown in Table 1. The amplification conditions were as follows: an initial denaturation of 95 °C for 15 min (to co-activate Hotstar *Taq* DNA polymerase), then 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min for 30 cycles, and a final extension of 72 °C for 10 min. Each primer set could be run individually using the same PCR amplification conditions.

In samples of mixed colonies with a positive PCR result, a pure-colony pick containing *aat*, *aaiA* or *astA* was obtained from the original MacConkey plate in all cases as follows. Five single colonies from each plate were inoculated into nutrient broth and incubated at 37 °C for 4–6 h. The single colonies were then examined for *aat*, *aaiA* and *astA* genes using the multiplex PCR, as described above.

Characterization of the bacterial strains using the HEP-2 adherence assay. All single-colony isolates were examined by the HEP-2 cell-adherence assay described by Nataro *et al.* (1987), with slight modifications. HEP-2 cells were grown for 24 h to 50–70% confluence on 13 mm diameter coverslips in 24-well flat-bottomed tissue-culture plates in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, 2% L-glutamate and 1% non-essential amino acids (Sigma-Aldrich). Prior

Table 1. Primers used in this study

Gene	Primer	Size of product (bp)
<i>aat</i>	5' CTG GCG AAA GAC TGT ATC AT	630
	3' CAA TGT ATA GAA ATC CGC TGT T	
<i>aaiA</i>	5' CTC TTA GCA GGG AGT TTG TC	430
	3' GCT TTG TTT ACC GAC TGA AC	
<i>astA</i>	5' CCA TCA ACA CAG TAT ATC CGA	111
	3' GGT CGC GAG TGA CGG CTT TGT	

to inoculation with bacterial strains, the HEP-2 cells were washed and 1 ml fresh DMEM medium with 1 % D-mannose (Sigma-Aldrich) was added. Isolates were recultured from Dorset's egg slopes to MacConkey agar and subcultured into LB broth culture, before overnight incubation without shaking at 37 °C. Twenty microlitres of the overnight LB broth culture were added to each well, and plates were incubated at 37 °C in an atmosphere of 95 % O₂/5 % CO₂ for 3 h. The HEP-2 cells were washed thoroughly with PBS to remove non-adherent bacteria and fixed with 70 % methanol for 30 min. After fixation, the coverslips were washed five times with PBS, stained with 10 % Giemsa (BDH) for 15 min, rinsed and air-dried, before mounting in DPX mountant (BDH) prior to light-microscopy examination using a ×40 objective. The adherence patterns were assessed and recorded (Fig. 1). When the 3 h incubation showed no adherence or an equivocal pattern of adhesion, a 6 h HEP-2 adherence assay was performed.

Serotyping. Each isolate was biochemically confirmed as *E. coli* and serotyped by the LEP, using a scheme based on the heat-stable somatic (O) and flagellar (H) antigens (Gross & Rowe, 1985). Strains that could not be serotyped using the LEP serotyping scheme were designated O?, O-rough or H?. Rough strains do not express an O antigen and therefore cannot be typed using a phenotypic serotyping scheme.

RESULTS AND DISCUSSION

Strains of *E. coli* harbouring the *aat*, *aaiA* or *astA* genes were found in faecal samples from 39 (7.8 %) of 500 patients. Sixteen of these isolates had the *aat* gene, the *aaiA* gene was found in 14 isolates and 29 strains had the *astA* gene. Twenty of the 38 strains adhered to the HEP-2 cells in a stacked-brick formation. A wide range of serotypes was detected, although 12 of the 36 strains could not be serogrouped in the current scheme and were designated O?. The details of these patients and the genotypes of their EAEC isolates are shown in Table 2.

Of the 16 patients that had isolates harbouring the *aat* gene, 12 also had the *aaiA* gene, eight carried the *astA* gene and 12 showed AA to HEP-2 cells in a stacked-brick pattern. *Campylobacter* species was also isolated from the stool of one

of these patients (Table 2). The *aat* gene PCR showed good correlation with the HEP-2 adhesion assay, as 75 % of *aat*-positive isolates were confirmed as EAEC. Baudry *et al.* (1990) first used the *aat* gene (referred to as the CVD432 EAEC probe) as a target for the identification of EAEC, and claimed that the probe was 89 % sensitive. Although subsequent studies have shown that the sensitivity varies between 15 and 90 % (Okeke & Nataro, 2001), the *aat* gene has remained the most popular target in molecular assays for the detection of EAEC (Schmidt *et al.*, 1995; Cerna *et al.*, 2003; Pabst *et al.*, 2003; Amar *et al.*, 2004). In this study, seven patients with *aat*-positive strains were known to have recently travelled outside the UK to destinations that included Nepal, Pakistan, Egypt, Sri Lanka and Mexico. Other studies have suggested an association between EAEC and travellers' diarrhoea (Adachi *et al.*, 2001; Wilson *et al.*, 2002).

Strains of *E. coli* harbouring the *aaiA* gene, but not the *aat* or *astA* genes, were isolated from two patients, one of which showed an aggregative phenotype. Twenty-one patients had strains of *E. coli* that carried the *astA* gene, but not the *aat* or *aaiA* genes. Seven of the 21 (33 %) strains adhered to HEP-2 cells in an aggregative pattern (Table 2). Other pathogens were isolated from four of these 21 faecal samples, including *Campylobacter* species (3) and rotavirus (1). A DA pattern was observed in two of the isolates that carried the *astA* gene, and one strain exhibited an LA pattern of adhesion (Table 2). Taking the adhesion assay as the gold standard, the inclusion of the primers detecting the *aaiA* and *astA* genes to the *aat* PCR enabled the assay to identify eight additional EAEC isolates and 12 isolates that were not EAEC.

The *astA* gene was once considered characteristic of the EAEC group (Savarino *et al.*, 1991), although it has since been found in only a subset of EAEC and has a broad distribution among other pathogenic and non-pathogenic *E. coli*. (Savarino *et al.*, 1996; Zhou *et al.*, 2002). Other workers have demonstrated that the prevalence of *astA*-positive *E. coli* in the stools of adults and children without diarrhoea can be as high as 38 % (Savarino *et al.*, 1996; Vila *et al.*, 1998). However, some *E. coli* strains isolated from humans with diarrhoea are found to harbour no known virulence factors other than EAST (Paiva de Sousa & Dubreuil, 2001; Nishikawa *et al.*, 1999). We cannot comment on the ability to cause diarrhoea of the strains that carried the *aat*, *aaiA* or *astA* genes but did not exhibit characteristic EAEC adherence, as this study was not designed to assess the role of these putative virulence factors or to determine whether strains harbouring these genes cause disease.

Using the HEP-2 adhesion assay as the gold standard, the addition of primers detecting *aaiA* and *astA* to the *aat* PCR increased the number of EAEC isolates detected, but identified strains of *E. coli* that were not EAEC. The variety of genotypes exhibiting AA highlights the problems associated with developing a molecular diagnostic test for EAEC. Our PCR assay detects a variety of strains exhibiting characteristics of the EAEC group, making it a useful tool for identifying both typical and atypical EAEC.

Fig. 1. HEp-2 cell adhesion assay showing the aggregative adhesion pattern. Bar, 10 μm.

Table 2. Patient details and combinations of *aat*, *aaiA* and *astA* in bacterial strains isolated from patients during this study

Abbreviations: F, female; M, male; N, no travel history; ND no data supplied; D, diarrhoea; V, vomiting; NT, strains not serotyped, as failed to grow on subculture.

Patient	Sex	Age	Travel	Consistency of sample	Clinical details	Other pathogens	<i>aat</i>	<i>aaiA</i>	<i>astA</i>	Adherence	Serotype
1	ND	ND	Pakistan	L	D	None detected	+	+	+	AA	O?:H16
30	F	A	Nepal	L	D	None detected	+	+	+	AA	O130:H?
226	F	A	N	L	D	None detected	+	+	+	AA	O51:H?
227	M	A	N	SF	D	<i>Campylobacter</i>	+	+	+	AA	O132:H25
333	M	A	N	SF	D+V	None detected	+	+	+	AA	O?:H16
67	F	A	N	F	D	None detected	+	+	+	No adherence*	O?:H2
34	F	A	Nepal	SF	D	None detected	+	+	–	AA	O?:H2
70	M	A	ND	SF	D	None detected	+	+	–	AA	O?:H?
221	ND	C	N	SF	D	None detected	+	+	–	AA	NT
400	F	A	Egypt	SF	D	None detected	+	+	–	AA	O?:H10
464	F	C	ND	SF	ND	None detected	+	+	–	AA	O?:H?
216	F	A	N	SF	ND	None detected	+	+	–	Not tested	NT
238	F	A	Sri Lanka	L	Gastroenteritis	None detected	+	–	+	AA	O1:H25
191	F	A	N	SF	Food poisoning	None detected	+	–	+	No adherence*	O134:H6
87	M	C	N	SF	D	None detected	+	–	–	AA	O?:H–
105	M	A	Mexico	SF	D	None detected	+	–	–	No adherence*	O?:H2
20	M	A	ND	SF	ND	None detected	–	+	–	AA	O77:H18
452	M	A	N	SF	D	None detected	–	+	–	No adherence	O?:H18
64	F	A	Palestine	SF	Stomach cramps	None detected	–	–	+	AA	O68:H12
75	M	A	Iran	SF	Stomach cramps	None detected	–	–	+	AA	O35:H–
134	M	A	N	L	D	None detected	–	–	+	AA	O17:H9
198	M	A	ND	SF	ND	None detected	–	–	+	AA	O169:H9
375	M	A	ND	SF	Gastroenteritis	None detected	–	–	+	AA	O?:H25
404	M	A	N	SF	D, abdominal pain	None detected	–	–	+	AA	O6:H?
408	F	A	N	SF	D+V	None detected	–	–	+	AA	O2:H56
131	F	A	South-East Asia	SF	D, fever	None detected	–	–	+	DA	O169:H9
269	M	C	N	SF	D	None detected	–	–	+	DA	O139:H56
265	F	A	ND	SF	D	None detected	–	–	+	LA	NT
50	M	A	N	SF	D	<i>Campylobacter</i>	–	–	+	No adherence	O36:H?
74	M	A	Peru	SF	D	<i>Campylobacter</i>	–	–	+	No adherence	O16:H16
149	F	A	N	F	Abdominal pain	None detected	–	–	+	No adherence	O133:H19
232	M	A	N	SF	D	None detected	–	–	+	No adherence	O41:H10
233	M	C	N	SF	D+V	None detected	–	–	+	No adherence	O?:H–
239	F	A	ND	SF	ND	None detected	–	–	+	No adherence	O148:H28
286	F	A	N	SF	D+V	None detected	–	–	+	No adherence	O2:H5
339	M	C	N	L	D+V	Rotavirus	–	–	+	No adherence	O41:H?
340	M	A	N	SF	Gastroenteritis	None detected	–	–	+	No adherence	O8:H7
390	M	A	ND	L	D	<i>Campylobacter</i>	–	–	+	No adherence	O36:H?
472	F	A	ND	SF	ND	None detected	–	–	+	No adherence	O86:H?

*PCR positive for *aat* gene but no AA observed.

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